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Solvent, Drying Time, and Substrate Affect the Responses of Lone Star Ticks (Acari: Ixodidae) to the Repellents Deet and Picaridin

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ABSTRACT Behavioral bioassays remain a standard tool in the discovery, development, and registration of arthropod repellents. Tick repellent bioassays are generally uncomplicated, but their results can be affected by basic variables (e.g., dimensions of testing materials, substrate, timing, temperature) of the assay. Using lone star tick, *Amblyomma americanum* (L.), nymphs in climbing bioassays, we tested for the effects of substrate, solvent, and drying time on tick responses. In dose–response tests, the widely used repellents *N,N*-diethyl-3-methyl benzamide (deet) and 1-methyl-propyl-2-(hydroxyethyl)-1-piperidinecarboxylate (picaridin) were applied to filter paper strips and challenged by ticks at 10, 20, 30, 40, and 120 min after application. At 10-min drying time, repellency at the intermediate concentration 500 nmol repellent/cm² filter paper was significantly lower for ethanol solutions of deet and picaridin (0 and 10% ticks repelled, respectively) than for solutions of deet and picaridin in acetone (96.7 and 76.7% ticks repelled, respectively). Repellency was greatest for both the acetone and ethanol solutions of deet and picaridin when challenged 120 min after application, and at shorter drying times at the highest concentration tested (2,000 nmol compound/cm²). The repellency of picaridin relative to deet differed at some combinations of solvent and drying time but not others. In dose–response tests using different paper substrates and a drying time of 10 min, both ethanol and acetone solutions of deet differed in repellency, depending on both the paper substrate and the solvent. However, there were no differences in repellency between ethanol and acetone solutions of deet applied to nylon organdy in an in vitro and in an in vivo (fingertip) bioassay. When deet in solution with various proportions of ethanol:water was applied at 2,000 nmol deet/cm² filter paper, the proportion of ticks repelled decreased as the proportion of water in the test solutions increased. Somewhat similar results were seen for solutions of deet in an acetone solvent. Water absorbed from the atmosphere may affect the efficacy of repellents in solution with anhydrous ethanol. Overall, results obtained from bioassays that differ in seemingly minor ways can be surprisingly different, diminishing the value of comparing studies that used similar, but not identical, methods. Nylon organdy or another similar thin cloth may be preferable to filter papers and copier paper for minimizing solvent-related differences. When a paper substrate is used, acetone may be the more suitable solvent if the solubility of the test compound and other factors allow.

KEY WORDS *Amblyomma americanum*, acetone, ethanol, water

Tens of thousands of new cases of tick-borne ailments, such as Lyme disease, human monocytic ehrlichiosis, and Rocky Mountain spotted fever infect Americans annually. Repellents are considered an effective means of personal protection against tick bite, and their use is recommended by the Center for Disease Control and Prevention (CDC 2002, Vazquez et al. 2008, Vaughn and Meshnick 2011). For decades, *N,N*-diethyl-3-methyl benzamide (deet) and permethrin have dominated the repellent market for uses on human skin and clothing, respectively (Schreck et al. 1982, Bissinger and Roe 2010). However, an interest by

the public in alternative repellents, particularly natural products, has added impetus to the search for new, effective, safe, and affordable repellents.

A variety of methods have been used in behavioral bioassays to measure the efficacy of tick repellents intended for use on human skin (Dautel 2004, Bissinger and Roe 2010, Pages et al. 2014). Typically, tick repellent bioassays involve the use of a solvent to dispense desired concentrations of the active solute evenly on a substrate. Papers (e.g., filter paper [Ndungu et al. 1995, Dautel et al. 1999, Carroll et al. 2004, Mehlhorn et al. 2005, Schwantes et al. 2008, Bissinger et al. 2009], recycled bond paper [Weldon et al. 2011]), and fabrics (e.g., cotton [Jaenson et al. 2006], nylon organdy [Carroll et al. 2005]) often are used as substrates, but other absorbent materials, such as cotton swabs (Dietrich et al. 2006), have also been used to receive repellent solutions. Time is usually allowed

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for the solvent to evaporate before test organisms are exposed to the repellent. When testing solutes with high vapor pressures, there is an incentive to start challenging the treatment while repellent activity is still detectable. Deet is considered the unofficial standard against which other repellent compounds are compared (Moore and Debboun 2007). Ethanol is the preferred solvent for deet (Qui et al. 1998).

The lone star tick has gained increasing attention as a nuisance biter and vector of rickettsial diseases such as human monocytic ehrlichiosis (Childs and Paddock 2003, Goddard and Varela-Stokes 2009, Stromdahl et al. 2011, Stromdahl and Hickling 2012). Active host seekers, lone star ticks, *Amblyomma americanum* (L.), are attracted to CO₂, readily leaving questing sites to move toward potential hosts (Wilson et al. 1972, Waladde and Rice 1982). Compared with blacklegged ticks, *Ixodes scapularis* Say, Armstrong et al. (2001) reported that host-seeking *A. americanum* are highly noticeable to the public. Its importance as a disease vector and its broad and expanding (Ginsberg et al. 1991) distribution across the south central, southeastern, and mid-Atlantic states northward to southern New England, warrant the inclusion of *A. americanum* in repellent studies.

After observing some unusual differences in routine bioassay results and using ethanol and acetone from several sources to prepare test solutions, Carroll and Kramer (2012) demonstrated that, for a wide range of concentrations, solutions of deet in acetone were greatly more repellent to nymphal *A. americanum* than solutions in ethanol when applied to filter paper 10–12 min before exposure to ticks in a climbing bioassay. Such differences have implications in repellent discovery and registration, and warrant further characterization. However, because only a single wait (drying time) in one kind of test with one repellent (deet) was reported, it is unclear whether this solvent effect generalizes to other tests.

To determine whether these solvent effects were important with other drying times, substrates, bioassays, and another repellent, we performed a variety of trials. We tested three concentrations of deet and the repellent 1-methyl-propyl-2-(hydroxyethyl)-1-piperidinecarboxylate (picaridin), both in ethanol and acetone solutions, at several drying times against *A. americanum* nymphs. The ethanol and acetone solutions of deet were tested on two types of filter paper, ordinary copier paper, and nylon cloth; substrates with different absorbencies.

We also tested *A. americanum* nymphs in a different system, using a fingertip bioassay and nylon cloth. Like the vertical filter paper bioassay, the fingertip bioassay exploits the tendency of host-seeking ticks to climb by confronting them with a repellent barrier, but in the fingertip bioassay, the ticks start on a host and must cross repellent-treated cloth to progress up the vertical finger. In both bioassays, the ticks are exposed to chemical, visual, and vibratory cues associated with the proximity of the experimenter.

Materials and Methods

Ticks. Nymphal *A. americanum* from a colony at Oklahoma State University, Stillwater, OK, were used in all tests except in experiment 5 in which we used *A. americanum* nymphs from USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, Kerrville, TX. The ticks were held at 23–24°C, ~97% RH, and a photoperiod of 16:8 (L:D) h. The *A. americanum* nymphs were tested 3–6 mo after molting.

Chemicals. Deet (purity 97%) was purchased from Aldrich, Sigma-Aldrich, Inc., St. Louis, MO. Picaridin (purity 98.7%) was obtained from LANXESS Corporation, Pittsburgh, PA. Acetone was purchased from Fisher, Fair Lawn, NJ, and anhydrous 200-proof ethyl alcohol from Warner-Graham, Inc., Cockeysville, MD.

Experiment 1: Vertical Paper Bioassays—Wait Time and Repellent Differences. We used an in vitro bioassay, described in detail by Carroll et al. (2004), that exploits the tendency of host-seeking *A. americanum* to climb. Briefly, a 4- by 7-cm rectangle of Whatman No. 4 qualitative filter paper (Whatman International, Maidstone, England) was marked with a pencil into two 1- by 4-cm zones at the far ends of the paper and a central 4- by 5-cm zone. Using a pipettor, 165 μ l of test solution was applied evenly to both sides of the central 4 by 5 cm of the paper, and the strip was balanced on the rim of a glass petri dish (9 cm in diameter) to dry. After drying (drying time [wait time] was an experimental variable, see below), the paper strip was suspended from a bulldog clip hung from a slender horizontal dowel held by an Aptex No. 10 double clip work holder (Aptex, Bethel, CT). A petri dish (9 cm in diameter) glued in the center of a 15-cm petri dish created a moat when water was added between their walls (1.5 cm in height). The moated petri dishes were placed beneath the suspended paper to confine ticks that dropped from the paper. When *A. americanum* nymphs had climbed to the rim of a storage vial opened in the center of moated petri dishes (5.5 and 9 cm in diameter), the paper strip was removed from the dowel and held so that 10 ticks crawled onto the lower untreated zone. Only ticks that readily transferred to the paper were used. The locations of the *A. americanum* nymphs were recorded at 15 min after the 10th nymph began clinging to the lower untreated zone of the paper. Ticks were considered repelled if they were in the lower untreated zone at 15 min or if they fell from the paper without having crossed the upper boundary of the treated zone.

Acetone and ethanol solutions of 125, 500, and 2,000 nmol deet or picaridin/cm² filter paper were applied to Whatman No. 4 filter paper strips and allowed to dry for 10, 20, 40, and 120 min before exposure to *A. americanum* nymphs. Three replicates of 10 nymphs each were tested for each compound–concentration–solvent–time combination. One control (10 ticks) of ethanol and acetone was tested each day of testing.

Experiment 2: Vertical Paper Bioassays—Substrate Differences. Whatman No. 1 and No. 4 qualitative filter papers and copier paper (92 bright, 75 g/m²,

Staples, Inc., Framingham, MA) were treated with acetone and ethanol solutions of 125, 500, and 2,000 nmol deet/cm² paper, all with a 10-min drying time, and used in the vertical bioassay described in experiment 1. Three replicates of 10 *A. americanum* nymphs each were tested for each compound-solvent-time combination. One control (10 ticks) of ethanol and acetone was tested each day of testing.

Experiment 3: Vertical Bioassays—Filter Paper Versus Cloth. The methods described in Experiment 1 for vertical paper bioassays were used for comparing tick responses to acetone and ethanol solutions, applied to Whatman No. 4 filter paper and nylon organdy (7 by 7 mesh/mm) (G Street Fabrics, Rockville, MD). A 4-by-7-cm rectangle of organdy, marked into treated and untreated zones like the filter paper strips, was held tautly and horizontally between two bulldog clips. Test solutions were applied between the pencil lines and spread evenly on upper and lower surfaces with the side of the pipette tip. The organdy strip was allowed to dry while affixed to the bulldog clips. Whatman No. 4 filter paper and organdy received either acetone and ethanol solutions of 0 (control) or 1,000 nmol deet/cm² of substrate and were allowed to dry for 10 min. Three replicates of 10 nymphs each were tested for each concentration-solvent-substrate combination. One control (10 ticks) of ethanol and acetone was tested each day of testing.

Experiment 4: Fingertip Bioassay. A version of the double-wrapped fingertip bioassays described by Carroll et al. (2005) in which no test solution is applied to the skin was conducted in compliance with a human-use protocol (no. 2007-240) reviewed and approved by the MedStar Research Institute Institutional Review Board. A strip of organdy was cut in the shape of a hockey stick (9-cm-long section, 4.5-cm-short section, 4–4.5 cm wide). The boundary of an area of the cloth corresponding to the area between the first and second joints of the finger was marked with a lead pencil and received the test solution. The volume of the solution applied to the cloth was based on the dimensions of the left index finger of J.F.C. The volume required for the desired nmol/cm² cloth was calculated from the average of the circumferences of the two finger joints multiplied by distance between the deepest crease of each joint.

While an organdy strip was partly supported by the rim of a glass petri dish, 52 μ l of test solution was evenly distributed on the treatment area with a pipettor. After allowing 10 min for the cloth to dry, it was doubly wrapped around the index finger of J.F.C., so that the treated portion of the cloth was only on the outer layer and completely encircled the finger covering the entire second phalanx. An untreated portion of the cloth extended 5–6 mm beyond the first joint toward the base of the finger. To hold the cloth in place, three small dabs of beeswax were smeared on the upper surface of the inner layer of cloth where layers overlapped, and pressure by another finger was applied to adhere the layers of cloth. Ten *A. americanum* nymphs were allowed to crawl from the rim of

an open vial (in a moated petri dish) onto the fingertip. Once 10 ticks were on the fingertip, the finger was tilted slowly until vertical with the tip downward. The locations of the ticks were recorded at 15 min after the 10th tick was on the finger. Ticks were considered repelled if they fell from the finger without having crossed the upper boundary of the treated area of cloth or if they were on the untreated fingertip distal to the cloth at 15 min. Before each bioassay J.F.C. washed his index finger with soap and rinsed with water. In the fingertip bioassay, 31.3, 46.9, 62.5, 125, and 500 nmol deet/cm² filter paper were tested with three groups (five groups for 46.9) of 10 nymphs at a 10-min drying time. One control (10 ticks) of ethanol and acetone was tested each day of testing.

Experiment 5: Deet in Ethanol-Water and Acetone-Water Solutions. Solutions of deet in 90:10, 80:20, 70:30 ethanol:water (deionized) and in ethanol alone and in 80:20 acetone:water and in acetone alone were applied at 2,000 nmol deet/cm² paper to Whatman No. 4 filter paper and allowed to dry for 10 min. Three replicates of 10 *A. americanum* nymphs were tested in the vertical bioassay described in experiment 1 for each combination of deet-solvent solution. One control (10 ticks) of ethanol and acetone was tested each day of testing.

Evaporation of Acetone and Ethanol From Filter Paper. A 5-by-7-cm strip of Whatman No. 4 filter paper was weighed on a Mettler AE100 balance (Mettler-Toledo, LLC, Columbus, OH) and the weight recorded. One hundred sixty-five microliters of acetone or ethanol were applied to the 4-by-5-cm central section of the strip, as in Experiment 1. The solvent was applied to the strip ~10 cm adjacent to the open side of the balance, so that it could quickly be placed on the balance. As soon as the application was completed, the strip was placed on the balance, simultaneous with activating a stopwatch. The weight of the strip was recorded almost immediately after the strip was placed in the balance and the sliding door shut (~2 s after application done). Weights were recorded at 1–10, 15, and 20 min after application. The sliding doors on the balance were left open until 10 s before each reading was taken, after which they were reopened. Five strips each were weighed for acetone and ethanol.

Statistical Analysis. The binomial data were fit using a generalized linear mixed model, with day as a random effect, using the R software (R Development Core Team 2012) and the lme4 package (Bates et al. 2011), with the default logit link. Although the day-to-day variance was not large (estimated to be <1.0), it accounted for most of the overdispersion observed in the data. Concentration-solvent combinations were fit as single 1 df fixed effects; a *P* value is reported for the appropriate contrast (for a *Z* statistic). Models involving different substrates and repellents were constructed similarly. A posteriori means comparisons were performed using the multcomp package (Hothorn et al. 2008).

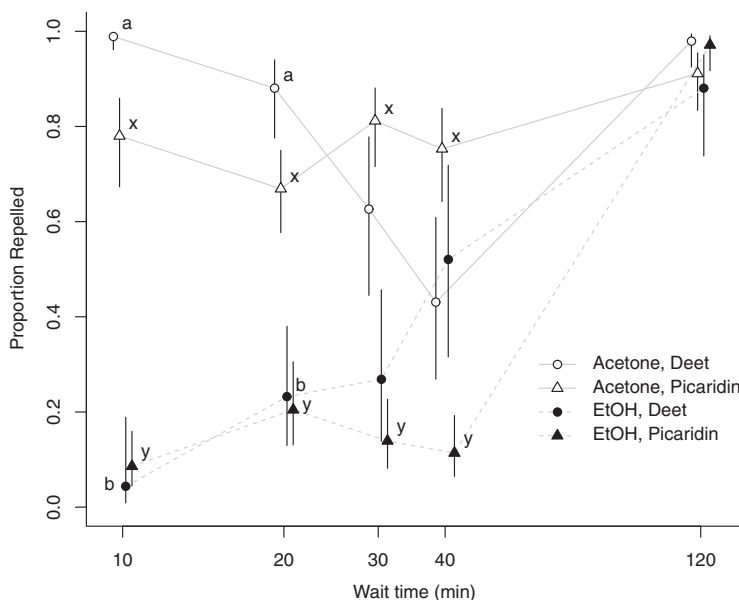


Fig. 1. Proportion of *A. americanum* nymphs repelled by deet and picaridin at 500 nmol compound/cm² at 10, 20, 40, and 120 min after the test solutions were applied to Whatman No. 4 filter paper in a vertical bioassay. For each wait time, if there were significant solvent differences between the proportions repelled by deet, an "a" is placed next to the acetone solution and a "b" next to the ethanol solution. For picaridin, the (x, y) letters were used. The (mostly) horizontal gray lines are used as a visual aid to connect the same repellent-solvent combination over wait time. The vertical black lines depict one standard error above and below the mean (these are asymmetric because they have been back-transformed from the logit scale).

Results

Experiment 1: Vertical Filter Paper Bioassays—Post-application (Wait/Drying) Time and Repellent Differences. There were effects of both solvent and postapplication (wait/drying) time (time between applying the solutions and testing, when the solvents were allowed to evaporate), but no differences in efficacy were observed between deet and picaridin (tested separately for each solvent, and for wait times of 20, 30, and 40 min, all $P \geq 0.20$), i.e., the solvent and wait time effects were similar for both repellents.

As seen in Fig. 1 (depicts results only for 500 nmol/cm², where solvent differences were greatest), the

repellents in an acetone solvent showed consistent high activity. The same repellents in the ethanol solvent attained their highest activity only after a wait period of 120 min; at short wait times, there was very little repellent activity (significant differences in the proportion repelled for deet in acetone versus deet in ethanol for each wait time are shown in Fig. 1 with the a–b letters, for picaridin with the x–y letters). Multiple comparisons tests for differences (contrasts) in wait times and their standard errors for 500 nmol compound/cm² are given in Tables 1 (deet) and 2 (picaridin).

In Table 1, for deet in the ethanol solvent, shorter wait times were not significantly different from the

Table 1. Multiple comparison results (error rate corrected) for contrast estimates and their standard errors (in parentheses) on the logit scale for *A. americanum* nymphs tested at 10, 20, 30, 40, or 120 min after applying 500 nmol deet/cm² to the Whatman No. 4 filter paper strip in a vertical bioassay

| Wait time (min) | Wait time (min) | | | | |
|-----------------|-----------------|--------------|--------------|--------------------------|---------------|
| | 10 | 20 | 30 | 40 | 120 |
| 10 | | 2.51 (1.36) | 3.99 (1.39)* | 4.79 (1.31) [†] | 0.64 (1.87) |
| 20 | –1.89 (1.70) | | 1.48 (0.81) | 2.27 (0.93) | –1.87 (1.55) |
| 30 | –2.09 (1.71) | –0.20 (1.01) | | 0.80 (0.85) | –3.35 (1.54) |
| 40 | –3.17 (1.78) | –1.28 (0.87) | –1.08 (1.15) | | –4.14 (1.53)* |
| 120 | –5.08 (1.89) | –3.19 (1.19) | –3.00 (1.27) | 1.92 (1.29) | |

These are contrasts of the proportion repelled (on the logit scale) between two wait times given as the row value minus the column value (so a positive contrast value results if the proportion of ticks repelled at the row wait time was larger than the proportion of ticks repelled at the column wait time, for negative values the proportion repelled at the column wait time are larger than that for the row wait time). Upper right triangle: acetone solvent, lower left triangle: ethanol solvent.

Means (on the original proportion scale) involved in each contrast are depicted in Fig. 1.

*Follows contrasts that were significant at $P < 0.05$.

[†]Contrasts significant at $P < 0.01$.

Table 2. Multiple comparison results for 500 nmol picaridin/cm² on the Whatman No. 4 filter paper strip in a vertical bioassay

| | Wait time (min) | | | | |
|-----------------|---------------------------|---------------------------|---------------------------|---------------------------|--------------|
| | 10 | 20 | 30 | 40 | 120 |
| Wait time (min) | | | | | |
| 10 | | 0.56 (0.57) | -0.20 (0.75) | 0.15 (0.66) | -1.06 (0.90) |
| 20 | -1.01 (0.85) | | -0.76 (0.64) | -0.41 (0.59) | -1.63 (0.82) |
| 30 | -0.54 (0.91) | 0.46 (0.73) | | 0.35 (0.75) | -0.87 (0.90) |
| 40 | -0.31 (0.88) | 0.70 (0.78) | 0.23 (0.85) | | -1.22 (0.89) |
| 120 | -5.89 (1.32) [†] | -4.88 (1.24) [†] | -5.34 (1.26) [†] | -5.57 (1.27) [†] | |

See Table 1 footnotes for further explanation.

120-min wait time (bottom line of lower left triangle), although two barely exceeded the $P = 0.05$ cutoff (even though they might appear to be different in Fig. 1). The reason for this appears to be the relatively large standard errors for these proportions (sample sizes were somewhat smaller).

At a concentration of 125 nmol compound/cm², the pattern was similar to that of 500 nmol compound/cm², though the proportion repelled at 120 min only reached 0.5 (not shown). At a concentration of 2,000 nmol compound/cm², repellent activity was high for all wait times and solvents (Fig. 2; note that proportion repelled, on the y-axis, starts at 0.5 when comparing with Fig. 1, where it starts at 0.0).

Experiment 2: Vertical Paper Bioassays—Substrate Differences. The different papers yielded different proportions repelled (Fig. 3). Whatman No. 4 filter paper and copier paper behaved similarly (high proportion of ticks repelled) for the acetone solvent, except at the 125 mol deet/cm² concentration where repellency was greater with copier paper. The proportions repelled using ethanol solutions of deet for these two substrates were similar except at the highest concentration, where more ticks were repelled for

copier paper (however, this is only borderline significant, $P = 0.078$). Whatman No. 1 filter paper treated with ethanol solutions of deet tended to be weakly repellent. The three significant a posteriori tests of paper differences (within a concentration and solvent, a total of 21 within concentration-solvent multiple comparisons were made) were copier versus Whatman No. 1 filter paper (125 nmol deet/cm², acetone), copier versus Whatman No.1 filter paper (500 nmol/cm², acetone), and copier versus Whatman No. 4 filter paper (500 nmol deet/cm², acetone); there were no significant differences among the papers using the alcohol solvent.

Results for comparing solvents follow. Deet dissolved in the acetone solvent clearly repelled a higher proportion of ticks than it did in ethanol at the 500 and 2,000 nmol/cm² concentrations (three paper types involved in the contrast for 500 nmol/cm², $P < 0.05$; however, only Whatman No.1 filter paper was used for the test at 2,000 nmol deet/cm² because data from the other two paper types, with 100% repelled, were not used when fitting the model, for this test $P < 0.05$).

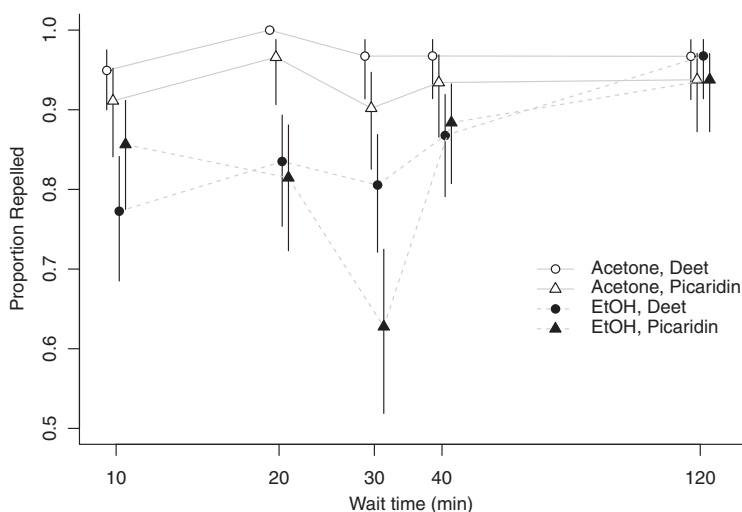


Fig. 2. Proportion of *A. americanum* nymphs repelled by deet and picaridin at 2,000 nmol compound/cm² at 10, 20, 40, and 120 min after the test solutions were applied to Whatman No. 4 filter paper in a vertical bioassay. The horizontal gray lines are used as a visual aid to connect the same repellent-solvent combination over wait/drying time, the vertical black lines depict one standard error above and below the mean. Note that the Y scale starts at 0.5 (when comparing with Fig. 1, where it starts at 0.0).

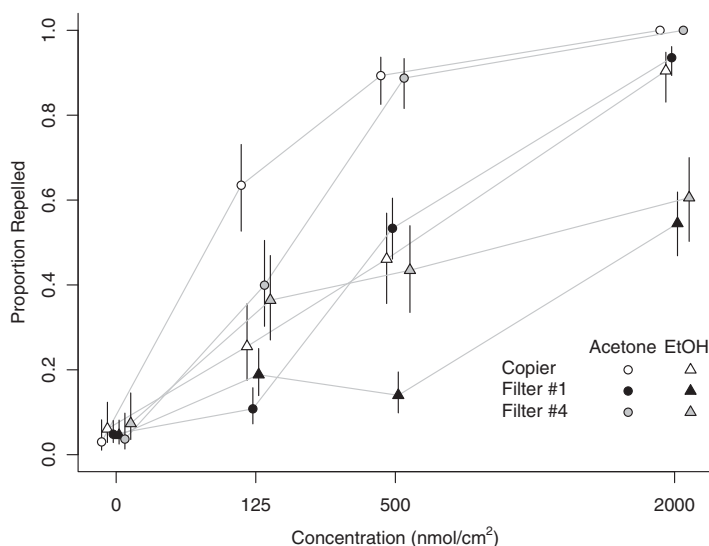


Fig. 3. Proportion of ticks repelled in vertical bioassay for four concentrations of deet in acetone or ethanol (EtOH), and applied to three different paper types. Drying time was 10 min. Vertical bars give a 67% confidence region (equivalent to one standard error) around the means. The confidence region is asymmetric because the model is estimated on the logit scale. Estimates and the confidence region are back-transformed to the proportion scale for easier interpretation. Means based on the same paper type and solvent are connected with gray lines.

Experiment 3: Vertical Bioassays—Filter Paper Versus Cloth. Ethanol solutions of 1,000 nmol deet/cm² applied to nylon organdy repelled 100% of the *A. americanum* nymphs, but when applied to Whatman No. 4 filter paper only 43.3% of the ticks were repelled (significant substrate difference, $P < 0.05$). However, there were no substrate differences ($P > 0.05$) for the acetone solvent at 1,000 nmol deet/cm² (96.7 and 100% of the ticks repelled, respectively). The two substrates also did not differ in repellency at 0 nmol deet/cm² for either solvent ($P > 0.05$).

Experiment 4: Fingertip Bioassay. Not only were the solvent differences not significant at each concentration, mean repellency rankings of the two solvents switched back and forth with increasing concentration (results not shown). This is consistent with the comparison of filter paper and organdy cloth in vertical tests (experiment 3, above), with little difference in repellency for the two solvents. A deet concentration effect in this assay was observed (results not shown, they are similar to those above).

Experiment 5: Deet in Ethanol-Water and Acetone-Water Solutions. When deet in solution with 90:10, 80:20, 70:30 ethanol:water mixtures and ethanol alone was applied to filter paper at 2,000 nmol deet/cm², the proportion of ticks repelled decreased as the proportion of water in the test solutions increased (note: the concentration of deet was the same in all these solutions, the proportion of water varied) (Fig. 4). All solutions of deet in ethanol-water and ethanol alone repelled lower proportions of ticks than deet in acetone alone. Deet in 80:20 acetone:water was similarly ineffective (3.3% repelled) compared with deet applied at the same rate in acetone alone (96.7% repelled) (Fig. 4).

Evaporation of Acetone and Ethanol From Filter Paper. At 22–24°C and 42–45% RH, acetone evaporated more quickly than ethanol from the filter paper, with 0 to ~1 mg remaining in the paper at 10 min compared with ≥ 2.7 mg of ethanol. Fig. 5 shows that changes in weight of Whatman No. 4 paper filter paper strips continued for >10 min after application of ethanol, although the paper felt dry to the touch.

Discussion

Filter paper treated with ethanol and ethanol solutions of repellents were ostensibly dry and felt dry when touched at 10 min after application. However, the impression that all the ethanol had volatilized by 10 min may have been misleading (Fig. 5). Rocklin (1976) compared the solvent evaporation rates from different substrates, specifically filter paper and a smooth aluminum surface. Alcohols and water had much lower evaporation rates (compared with n-butyl acetate) from filter paper than compounds with the same vapor pressure, which Rocklin (1976) attributed to hydrogen bonding interaction of hydroxylic compounds with the cellulosic substrate.

A short drying and preexposure time may be necessary when tick responses to highly volatile chemicals are of interest. Short drying times may yield quicker results and allow more data to be generated over a shorter period of time than waiting ≥ 40 min after the application of a test solution to start bioassays. However, in general, prolonged postapplication activity is desirable in repellents, and well planned scheduling of treatments and exposures can maximize output of bioassays.

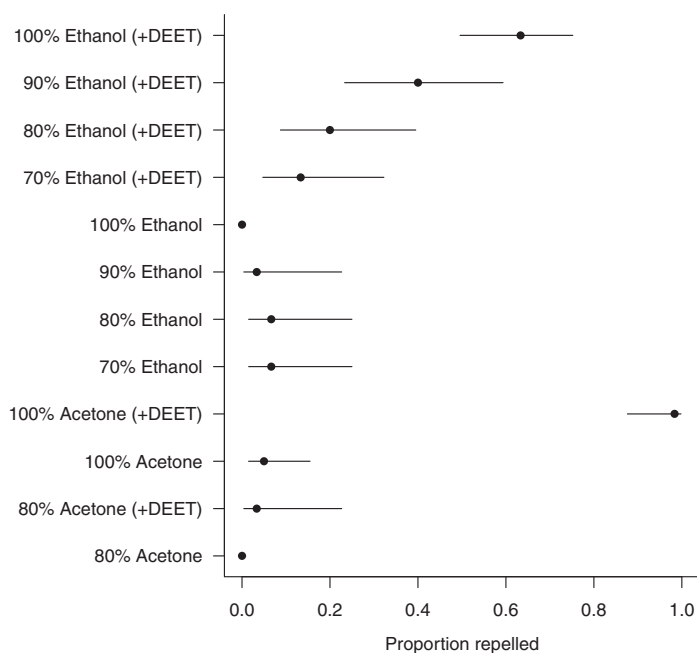


Fig. 4. The proportion of ticks (points) repelled for various ethanol and acetone solutions, with and without water and deet. Horizontal lines give $\sim 95\%$ confidence intervals on the proportions (not calculated for 0% repelled). Water accounts for the remaining percent of the solvent in y-axis labels. All deet treatments were applied at $2,000 \text{ nmol deet/cm}^2$. Testing on vertical strips was conducted after waiting 10 min.

Whatman No. 4 filter paper treated with ethanol clearly retained its weight longer than filter paper treated with acetone (Fig. 5), a difference quite visible at 10 min after application and still discernible at 20 min. However, experiment 1 showed that the repellency of deet and picaridin applied in ethanol solutions to Whatman No. 4 filter paper still was depressed at 30 and 40 min postapplication (Fig. 1). As the concentrations of deet and picaridin increased in a constant volume of test solution, so did the efficacy of the repellents at 10, 20, and 30 min.

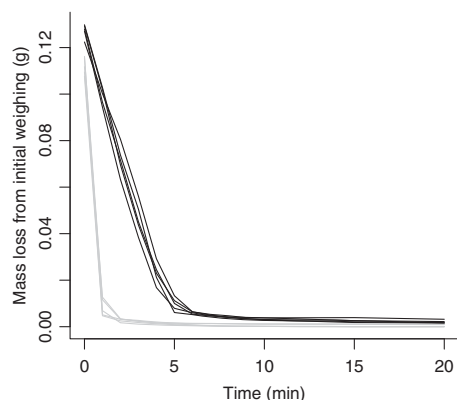


Fig. 5. Evaporation of $165\text{-}\mu\text{l}$ acetone (gray lines) and ethanol (black lines) from Whatman No. 4 filter paper ($n = 5$ for each solvent) as decrease in weight of the paper over 20 min postapplication, at $22\text{--}24^\circ\text{C}$ and $42\text{--}45\%$ RH.

If ethanol is an attractant to *A. americanum*, it was not clear from the bioassays. As with acetone controls, $>90\%$ of the ticks climbed from a lower untreated portion of the vertical filter paper strip through the ethanol-treated zone to an upper untreated portion of the paper. Presumably, if residual ethanol attracted the ticks, they would tend to remain on the treated portion of the filter paper. The volumes of test solutions we applied to the papers and the nylon organandy were sufficient for an even distribution throughout the designated treatment areas of the substrates (i.e., no paths of low concentrations of repellent allowing a tick to negotiate its way through the treatment area).

It is possible that tick repellent receptors were somehow blocked by ethanol or a denaturant that remained in the paper for 30 min, but experiment 5 showed a curious relationship between the presence of water in the test solutions and diminished repellency of ethanol and acetone solutions of deet. The anhydrous ethanol might absorb water from the atmosphere, which could linger and interfere with the exposure of deet or picaridin applications to ticks and may account for the continued slight additional weight of filter papers after a steep decline in weight in the first 5 min after application. The results of experiment 5 are consistent with water having a negative effect on repellency of *A. americanum*. Although the rate of deet applied per cm^2 was the same, as the proportion of water to ethanol increased in test solutions, fewer ticks were repelled. When water was combined with acetone (80 acetone: 20 water), re-

pellency was 3.3% for a concentration of deet that in acetone alone repelled 96.7% of *A. americanum* nymphs. Ultimately of interest is how water on skin might affect repellency of repellents, and if necessary, how formulations might counteract the effects observed in experiment 5. It is well known that dragging or flagging for host-seeking ticks is unproductive when the vegetation is wet. Kröber and Guerin (1999) showed that larvae of the tick *Rhipicephalus (Boophilus) microplus* (Canestrini), and all active stages of the tick *Ixodes ricinus* L., avoid walking on a membrane wet with water. Based on their observations, Kröber and Guerin (1999) suggested that the tick water receptors are in terminal pore sensilla on the tarsi of the forelegs. Perhaps water avoidance is not due to just the physical difficulties of ticks negotiating movements on wet stems and leaves. We observed that *A. americanum* nymphs tended to climb filter strips treated with 70 and 80% ethanol–water solutions with and without deet less rapidly and in a more circuitous manner than strips treated with acetone and ethanol alone.

Absorption of deet in ethanol solutions into skin has been investigated in some depth (e.g., Stineciper and Shah 1997, Qui et al. 1998, Santhanam et al. 2005). Stineciper and Shah (1997) recommended that alternative mosquito repellent formulations should be developed to decrease or prevent dermal absorption of deet. Ethanol has been used as a skin permeation enhancer for drug delivery (Ghosh and Banga 1993).

People usually apply repellents just before they enter tick habitat, or generally less than an hour in advance. Little is known whether commercial deet products are more, less, or equally effective against ticks 15 min after application than 1 h after application. However, as our results suggest, if the concentration of deet is sufficiently high, a postapplication time effect would not be noticeable.

Our findings indicate that investigators contemplating conducting repellent bioassays against ticks should consider the appropriateness of solvents, drying times, and substrates with respect to what information they wish to derive from the analyses. For instance, without a compelling reason to the contrary, short drying times and ethanol as a solvent should be eschewed. Less absorbent substrates, such as nylon organdy (Carroll et al. 2007), bond paper (Weldon et al. 2011), or glass (Kröber et al. 2010) might be substituted for filter paper, or filter papers of low absorbency used when ethanol is the solvent. Although our findings might appear to suggest that acetone should be the solvent of first choice for repellent bioassays, ethanol may be preferred in some cases for solubility reasons. Because ethanol is widely used in commercial formulations of deet and other repellents, it can be argued that ethanol solutions of test repellents are truer models of the repellent products that consumers buy.

The temporary inhibition of repellency of deet and picaridin and whether it is strictly an artifact of the test methods and materials or has wider implications, on mosquitoes for example, await explanation. Further investigation of the effects of the various materials used in repellent bioassays on test results is warranted.

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